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EXPRESSION OF THE *WT1* WILMS' TUMOR GENE BY NORMAL AND MALIGNANT HUMAN MELANOCYTES

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We report expression of the wt1 (Wilms' tumor) gene by cultured human melanoma cells. Using RNA polymerase chain reaction analysis, wt1 transcripts were detected in 7 of 9 melanoma cell lines but not in 5 normal melanocyte strains. In Northern blot analysis, steady-state wt1 mRNA levels were found in 2 of 4 melanoma lines but not in normal melanocytes. Sequence analysis of the wt1 cDNA expressed by melanoma cell line WM 902-B revealed the presence of 4 previously published splice variants but no evidence for mutations in the coding region. Previous work has shown that WT1 modulates transcription after binding to the early growth response (EGR)-1 sites present in the platelet-derived growth factor (PDGF)-A chain promoter; the PDGF-A chain gene is known to be expressed by various melanoma cell lines. Based on these findings, we studied the relationship of wt1 and PDGF-A chain gene expression in melanoma cell lines. Co-expression of the wt1 and the PDGF-A chain genes was observed in 2 melanoma cell lines with mutated *p53* but not in 2 melanoma cell lines with wild-type *p53*; this result is consistent with a previous report showing that, in the context of absent or mutated p53, WT1 acts as a transcriptional activator, whereas in the presence of wild-type p53 it acts as a repressor.

The *wt1* gene encodes a protein (WT1) with the structural features of a DNA-binding transcription factor. It contains 4 contiguous zinc fingers at the carboxy-terminus, and a glutamine- and proline-rich region at the amino-terminus (Call *et al.*, 1990; Gessler *et al.*, 1990). The zinc finger region has been shown to interact specifically with the early growth response (EGR)-1 DNA consensus sequence (Rauscher *et al.*, 1990; Wang *et al.*, 1992) and other less defined sequences in the platelet-derived growth factor (PDGF)-A chain promoter (Wang *et al.*, 1993), whereas the amino-terminal region of WT1 mediates transcriptional suppression or activation in transient transfection assays (Madden *et al.*, 1991; Maheswaran *et al.*, 1993). The *wt1* gene located on chromosome 11p13 is mutated or deleted in a subset of hereditary and sporadic Wilms' tumors, consistent with the notion that *wt1* represents a tumor suppressor gene in this system (Haber *et al.*, 1990). In contrast to some ubiquitously expressed tumor suppressor genes, such as the *rb1* retinoblastoma susceptibility and the *p53* gene, expression of *wt1* mRNA appears to be developmentally restricted (Pritchard-Jones *et al.*, 1990, 1991; Park *et al.*, 1993) and has been reported in mesodermally derived tissues and cell types including the urogenital system, spleen, mesothelial cells and brain. High levels of *wt1* expression were reported in tumors derived from these tissues such as ovarian carcinomas (Bruening *et al.*, 1993), mesotheliomas (Park *et al.*, 1993) and leukemias (Miwa *et al.*, 1992; Miyagi *et al.*, 1992), suggesting a broad range of *wt1* expression in malignant tissues.

In this study, we describe expression of the *wt1* gene in cultured human melanoma cells but not normal melanocytes. In addition, we investigated the relationship of *wt1* expression to the expression of the PDGF-A chain gene, which is a growth factor produced by some malignant melanoma cell lines (Westermark *et al.*, 1986; Albino *et al.*, 1991; Rodeck *et al.*, 1991). The PDGF-A chain promoter contains multiple WT1 DNA-binding sequences that have been shown to facilitate transcriptional regulation of PDGF-A chain gene expression by WT1 (Gashler *et al.*, 1992, Wang *et al.*, 1992, 1993); in transient co-transfection assays, WT1 in concert with wild-type p53 suppresses PDGF-A chain expression, whereas, in the context of absent or mutated p53, WT1 acts as a transcriptional activator of EGR-1 containing promoter constructs (Maheswaran *et al.*, 1993). Our results suggest that co-expression of the *wt1* and the PDGF-A genes in melanoma cells occurs in the presence of *p53* mutations.

MATERIAL AND METHODS

Cell lines

Establishment and routine tissue culture of the melanoma cells used in this study have been described previously (Herlyn *et al.*, 1985*a* and *b*; Kath *et al.*, 1991). WM 1158, WM 239-A, WM 1617, WM 9, WM 852, WM 164, WM 983-B and WM 373 were derived from metastatic melanomas, whereas WM 39, WM 902-B, WM 793 and WM 983-A were isolated from primary melanomas. WM 983-A and WM 983-B were isolated from the same patient; WM 983-A is derived from the primary, and WM 983-B from a lymph node metastasis. Normal melanocyte cultures were isolated from neonatal foreskins and maintained as described previously (Herlyn *et al.*, 1988).

RNA-polymerase chain reaction and hybridization analysis of amplification products

Primers used for amplification of *wt1*, PDGF-A chain, and β-actin sequences are shown in Table I. Logarithmically growing cells were lysed in guanidine isothiocyanate, and total cellular RNA was prepared using either cesium chloride gradient centrifugation as described (Rodeck *et al.*, 1991) or the RNAzol RNA extraction kit (Promega, Madison, WI). Reverse transcription (RT) was performed using 1 μg of total RNA dissolved in 8 μl H_2O, heated for 5 min at 65°C and chilled on ice. The RT reaction mixture of 20 μl contained 4 μl 5 × RT buffer (BRL, Gaithersburg, MD), 1 μl dNTP (10 mM of each; Pharmacia, Piscataway, NJ), 1.2 μl RNAsin (Promega), 1 μl oligo(dT) (50 μM; Perkin Elmer Cetus, Norwalk, CT) as the cDNA primer and 1 μl MMLV RT (BRL; 200 U/μl). The RT reaction was allowed to proceed for 30 min at 37°C and then stopped by heating to 98°C for 2 min.

Two different protocols were used to amplify *wt1* cDNAs. In the first protocol we used nested primers for the *wt1* zinc finger region, as shown in Table I; this protocol was used for *wt1* screening followed by Southern blot analysis of amplification products. Briefly, 5 μl of RT reaction product were added to 95 μl PCR mixture containing the first set of primers at 0.2 μM concentration, 10 μl 10× amplification buffer, 0.5 μl Taq DNA polymerase (Perkin Elmer Cetus) and dNTPs at 100 nM final concentration. Then PCR reaction mixtures were covered with mineral oil. For the first *wt1* zinc finger domain amplification the following temperature profile was chosen: 1 cycle at 95°C for 1 min, 30 sec, 42°C for 1 min and 72°C for 2 min followed by

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TABLE I - PRIMERS USED FOR PCR ANALYSES

RNA transcript	Location in cDNA sequence			
	5' oligonucleotide	3' oligonucleotide	Fragment size (bp)	Reference
β-actin	103–122	619–642	541	Ng *et al.*, 1985
*wt*1				Haber *et al.*, 1991[1]
Nested primers				
1st set	896–915	oligo dT		
2nd set	926–945	1,317–1,336	412/403	
Fragments				
1	814–833	1,393–1,412	608/599	
2	257–275	856–875	619/568	
3	107–126	296–315	209	
4	−21–−2	133–153	174	
PDGF-A chain	388–407	1,037–1,056	600/669[2]	Betsholtz *et al.*, 1986

[1]The A residue of the initiation AUG codon of *wt*1 is referred to as +1; the nucleotide numbering includes all splice variants, *i.e.*, exon 5 and +KTS.–[2]The PDGF-A chain cDNA sequence published originally by Betsholtz *et al.* (1986) includes the exon 6 sequence (69 bp); we could detect only a 600 bp amplification product consistent with the production of the physiological splice variant lacking exon 6 (Matoskova *et al.*, 1989) in human melanoma cells.

25 cycles at 95°C for 50 sec, 48°C for 1 min and 72°C for 1 min. 20 sec. The time extension was 3 sec, and the last step of the last cycle was delayed for 8 min. For the second *wt*1 zinc finger domain amplification we used 4 cycles at 95°C for 50 sec, 56°C for 1 min and 70°C for 1 min, time extension 2 sec followed by 26 cycles at 95°C for 50 sec and 70°C for 30 sec. The time extension for the latter 26 cycles was 3 sec, and the last step of the last cycle was delayed for 8 min. PCR products were electrophoresed on 2% agarose (BRL Ultrapure) gels and transferred to Zetabind nylon membranes for further analysis. For hybridization analysis we used a 3.5 kb *Eco*RI to *Hind*III fragment excised from β-actin cDNA cloned into pBR322 (Khalili and Weinmann, 1984), the WT33 fragment of *wt*1 cDNA (Call *et al.*, 1990) and an oligoprobe (WT-01; GACCCA-CACCAGGACTCATACAGG) hybridizing to part of the third zinc finger region of *wt*1 cDNA. Membranes were hybridized with either random prime labeled (multi-prime DNA; Amersham, Arlington Heights, IL) cDNA probes for *wt*1 (WT33) or β-actin or the end-labeled WT-01 oligonucleotide probe. Hybridization conditions and washing procedure for the WT-01 oligonucleotide probe were as described (Linnenbach *et al.*, 1983).

The second *wt*1 amplification protocol was used to amplify the entire *wt*1 cDNA for sequencing using primer sets that produce 4 overlapping fragments (Brown *et al.*, 1992). Reverse transcription and PCR amplification were performed as described (Brown *et al.*, 1992) with 2 modifications that were found to reduce unspecific PCR products significantly. We used the "hot start" technique (D'Aquila *et al.*, 1991) and 6% formamide in the PCR mix for the two 5' *wt*1 fragments or 4% formamide for fragment 2. Several independent cDNA preparations per primer set were sequenced using an automated DNA sequencer. Both strands of the PCR-generated *wt*1 cDNA fragments were either sequenced directly or after cloning into pUC19. At least 4 independent DNA preparations per primer set were analyzed to distinguish PCR-induced artefacts from pre-existing point mutations.

Northern blot analysis

Northern blots were performed essentially as described previously (Rodeck *et al.*, 1991). As an RNA loading control, we used a human cDNA probe for α-enolase (Giallongo *et al.*, 1986).

RESULTS

Expression of steady-state wt*1 mRNA by melanocytic cells*

We screened *wt*1 mRNA expression in melanoma cells compared with normal melanocytes using RNA PCR. The primers chosen flank the zinc finger region (nucleotides 623–1024) (Call *et al.*, 1990) and span several exon/intron boundaries. After PCR amplification, the amplified sequences were gel electrophoresed, blotted and hybridized with an oligonucleotide probe (WT-01) and the WT33 cDNA probe. A single, identical PCR product of approximately 400 bp was detected in material derived from 7 different melanoma cell lines (Fig. 1); the expected amplification products are 403 and 412 bp (Table I). The size of the PCR product is consistent with amplification of mRNA-derived cDNA but not genomic DNA due to the exon/intron structure of the *wt*1 gene (Morris *et al.*, 1991). Only one melanoma-derived PCR product was distinguishable in ethidium bromide-stained gels and after hybridization, indicating that no gross alterations were present in the zinc finger region of melanoma-derived *wt*1. No hybridization signal was detectable even after prolonged exposure of PCR products obtained from 2 melanoma line mRNAs (WM 239-A, WM 983-A), although β-actin sequences could easily be detected (Fig. 1). Similarly, no hybridization of either *wt*1 probe was detected in the *wt*1 PCR products obtained from 5 normal melanocyte strains (Fig. 2). Also, several RNA isolates from WM 983-A cells were consistently negative in *wt*1 RNA-PCR analysis. Control amplification of β-actin shows that comparable amounts of melanocyte and melanoma cell RNA were used for PCR amplification.

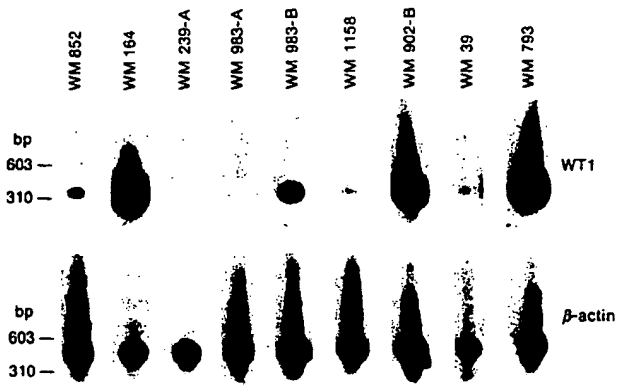

FIGURE 1 – Detection of PCR amplified *wt*1 zinc finger mRNA sequences in melanoma cells in culture as determined by Southern blot analysis of melanoma-derived RNA-PCR products using the ^{32}P-labeled WT33 cDNA fragment. Amplification products obtained using primers specific to β-actin sequences and hybridizing to β-actin cDNA probe are shown as controls. The exposure time for all autoradiographs shown was 3 hr.

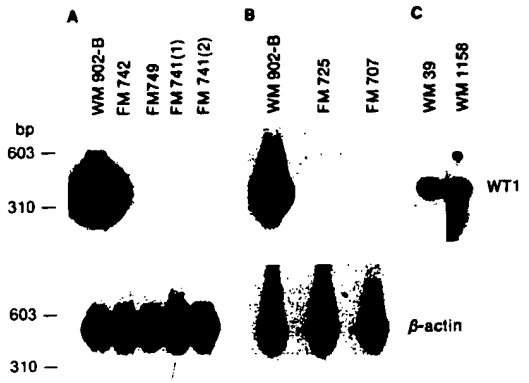

FIGURE 2 – Differential detection of PCR-amplified *wt*1 zinc finger mRNA sequences in normal melanocytes (FM 707, FM 725, FM 741, FM 742 and FM 749) compared with melanoma cells (WM 902-B, WM 1158 and WM 39). The results of 2 different experiments are shown in panels *a* and *b*. In the first experiment *(a)* the exposure time of autoradiographs after hybridization with the WT33 cDNA probe was 3 days, and after hybridization with the β-actin probe the exposure time was 3 hr. RNA from melanocyte strain FM 741 was isolated at two different occasions. Exposure times in the second experiment *(b)* were 3 days for *wt*1 and 1 hr for β-actin. For the purpose of comparison, *wt*1 signals after 3 days of exposure are shown for the melanoma lines WM 39 and WM 1158 *(c)* that have low-level *wt*1 expression, as shown in Figure 1.

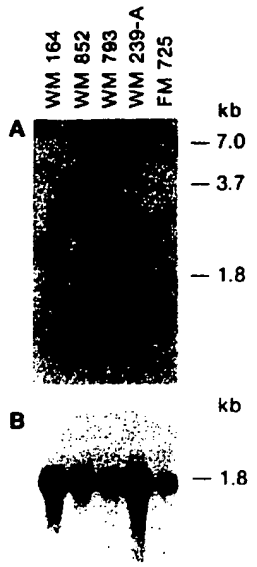

FIGURE 3 – Differential expression of *wt*1 transcripts in normal melanocytes (FM 725) and melanoma cells (all others) as determined by Northern blot analysis using the WT33 cDNA *(a)* probe. Hybridization with the α-enolase probe *(b)* is shown to demonstrate that comparable amounts of mRNA were loaded in each lane.

Northern blot analysis of melanoma and melanocyte poly(A)-enriched RNA using the WT33 cDNA fragment as a probe showed a single transcript of approximately 3 kb in melanoma cell lines WM 164 and WM 793 that tested positive in RNA-PCR (Fig. 3*a*). One RNA-PCR-positive cell line (WM 852) did not express *wt*1 mRNA at levels detectable in Northern analysis. Consistent with this finding, WM 852 expressed comparatively lower levels of *wt*1 mRNA when compared with WM 164 and WM 793 in RNA-PCR (Fig. 1). Melanoma cell line WM 239-A was negative in RNA-PCR and in Northern blot analysis. Similarly, no *wt*1 transcripts were detected in RNA isolated from melanocyte line FM 725, which was also negative in RNA-PCR analysis. No *wt*1 transcripts were detected by either probe when total RNA (10 μg/lane) was used in Northern blot analysis (results not shown).

Sequence analysis of wt*1 derived from melanoma cell line WM 902-B*

Overlapping primer sets were used to amplify 4 different fragments spanning the entire *wt*1 coding region expressed by melanoma cell line WM 902-B, which was chosen for this analysis because it expressed high transcript levels of *wt*1 as determined in the initial RNA-PCR screen (Fig. 1). Sequence analysis of these PCR fragments did not reveal any mutation of the *wt*1 gene in the coding region but demonstrated the presence of 2 physiological splice variants (Haber *et al.*, 1991). Splice I is characterized by the presence or absence of 51 nucleotides representing exon 5 of the *wt*1 coding sequence. Splice 2 results in the addition of 3 amino acids (KTS) between zinc fingers 3 and 4 (Splice I) (Haber *et al.*, 1991).

Co-expression of the wt*1 and the PDGF-A chain genes in melanoma cell lines*

To explore functional consequences of *wt*1 expression in melanoma cells we assessed coexpression of *wt*1 and the melanoma-associated PDGF-A chain gene. The PDGF-A promoter contains 2 overlapping EGR-1 consensus sites and was shown to be repressed by WT1 (Wang *et al.*, 1992, 1993). It has been observed previously that transcriptional repression of EGR-1 sites is not an intrinsic property of WT1 but depends on the presence of wild-type p53 (Maheswaran *et al.*, 1993). By contrast, p53 in the mutant conformation renders WT1 a transcriptional activator. We therefore focused this survey on 6 melanoma cell lines in which information on the expression of mutated or wild-type p53 was available from a previous study (Weiss *et al.*, 1993).

TABLE II – EXPRESSION OF WT1 AND PDGF-A BY MELANOMA CELL LINES

Cell line	WT1	PDGF-A[1]	p53[2]
WM 852	+	+	Mutated
WM 164	++	+	Mutated
WM 983-A	–	–	Mutated
WM 983-B	+	–	Mutated
WM 239-A	–	+	wt
WM 1158	(+)	–	wt

[1]Expression determined by RNA-PCR analysis followed by agarose gel electrophoresis and staining of amplification products with ethidium bromide.–[2]Data on p53 mutations affecting codon 220 (WM 164), codon 241 (WM 852) and codon 278 (WM983-A, WM 983-B) were derived from Weiss *et al.* (1993).

RNA-PCR analysis for both genes demonstrated no apparent relationship between *wt*1 and PDGF-A chain expression; in fact, co-expression of *wt*1 and PDGF-A chain mRNA was observed in 2 of 4 cell lines that express *wt*1 (Table II). However, both of these cell lines harbor *p53* mutations affecting codons 220 and 241, respectively, whereas the 2 cell lines that contain wild-type p53 do not co-express *wt*1 and PDGF-A chain mRNA.

DISCUSSION

Our study demonstrates expression of *wt*1 mRNA by approximately 70% of human melanoma cells cultured *in vitro*. By

contrast, Call *et al.* (1990) described expression of the *wt1* gene exclusively in human Wilms' tumor and hemopoietic cell lines, but not in cell lines derived from various carcinomas, sarcomas and, most relevant to our study, 2 melanomas. This discrepancy may be due to the fact that melanoma cells express low *wt1* steady-state mRNA levels detectable only in poly(A)-enriched melanoma RNA after prolonged exposure of autoradiographs. These levels are too low to be detected in Northern blot analysis of total RNA performed in the earlier study and are clearly lower than those observed in Wilms' tumor cell lines (Haber *et al.*, 1990; Brown *et al.*, 1992), leukemias (Miwa *et al.*, 1992, Miyagi *et al.*, 1992) and mesotheliomas (Park *et al.*, 1993), in which *wt1* transcripts were detected in total RNA preparations. The melanoma-derived *wt1* mRNA appears normal in size (approx. 3.0 kb) and comparable to the single *wt1* mRNA species of approximately 3 kb found in normal baboon kidney and normal human fetal kidney (Pritchard-Jones *et al.*, 1990; Pelletier *et al.*, 1991*b*).

In contrast to melanoma cells, cultured normal melanocytes did not express the *wt1* gene at levels detectable by either Northern blot analysis or the PCR procedure used in our study. A similar differential expression of *wt1* has been observed between adult normal and malignant kidney cells (Haber *et al.*, 1991). Expression of *wt1* mRNA was not restricted to end-stage metastatic melanoma cell lines but extended to cells derived from an earlier stage of tumor progression, *i.e.*, primary melanoma. Three of 4 primary (WM 902-B, WM 793 and WM 39) and 4 of 5 metastatic melanoma lines (WM 852, WM 164, WM 983-B and WM 1158) expressed *wt1* mRNA as determined in RNA-PCR analysis. Interestingly, the primary WM 983-A and the metastatic WM 983-B cell lines were isolated from the same patient but differed in *wt1* mRNA expression. Whereas primary WM 983-A cells did not express *wt1* mRNA at levels detectable in RNA-PCR, WM 983-B cells showed *wt1* expression, suggesting that, in this patient, expression of *wt1* was a late event in tumor progression.

In Wilms' tumors intragenic deletions and point mutations of *wt1* have been shown to result either in loss of function due to loss of DNA binding or change of function (Pelletier *et al.*, 1991*a*; Huff *et al.*, 1991; Little *et al.*, 1992; Haber *et al.*, 1993). In a mesothelioma cell line, a point mutation was identified that caused WT1 to act as a transcriptional activator of the EGR-1 site in transient co-transfection assays (Park *et al.*, 1993). By contrast, sequence analysis of *wt1* transcripts expressed in WM 902-B melanoma cells revealed no evidence for a point mutation in the *wt1* coding sequence. Whether this finding can be extrapolated to other melanoma cell lines remains to be determined. However, this result raises the question of the transcriptional role of WT1 in melanoma cells. We have begun to address this issue by comparing *wt1* expression with expression of a known target gene for WT1, the PDGF-A chain gene. Previous reports have shown that WT1 binds to 2 overlapping EGR-1 consensus sites in the PDGF-A chain promoter (Gashler *et al.*, 1992; Wang *et al.*, 1992). In co-transfection assays, wild-type WT1 represses transcription from the PDGF-A promoter. Based on these studies, we hypothesized that expression of the *wt1* and PDGF-A chain genes should be mutually exclusive in melanoma cells. This is clearly not the case, as shown by co-expression of both genes in melanoma cell lines WM 164 and WM 852. At present, it is unknown whether co-expression of these 2 genes in these cells is due to: 1) mutations in the *wt1* sequence; 2) alterations of the PDGF-A promoter; or 3) the modulation of WT1 function in those cells by other transcription factors such as p53. We favor the latter possibility, because both cell lines co-expressing *wt1* and PDGF-A chain have point mutations affecting codons 220 and 241 of the p53 sequence (Weiss *et al.*, 1993). A previous report has demonstrated that, in the presence of mutated rat p53, WT1 does not act as a transcriptional repressor but rather as a transcriptional activator of EGR-1 sites (Maheswaran *et al.*, 1993). The point mutations in melanoma cells WM 164 and WM 852 are at positions different from the point mutation already shown to alter WT1 transcriptional properties, which is the rat homologue of a human codon 248 mutation. However, it may be anticipated that all 3 mutations produce the "mutant conformation" of p53, which appears to be important for p53 function.

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